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3-Methylhistidine turnover in the whole body, and the contribution of skeletal muscle and intestine to urinary 3-methylhistidine excretion in the adult rat

David J. MILLWARD and Peter C. BATES
Clinical Nutrition and Metabolism Unit, Department of Human Nutrition, London School of Hygiene and Tropical Medicine, 4 St. Pancras Way, London NW1 2PE, U.K.

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The tissue origin of 3-methylhistidine (N^{τ}-methylhistidine) was investigated in adult female rats. The decay of labelling of urinary 3-methylhistidine was compared with the labelling of protein-bound 3-methylhistidine in skeletal muscle and intestine after the injection of [*methyl*-^{14}C]methionine. The decay curve for urinary 3-methylhistidine was much steeper than that in muscle or intestine, falling to values lower than those in either tissue after 30 days. The lack of decay of labelling in muscle during the first 30 days is shown to result from the persistence of label in the precursor *S*-adenosylmethionine. The relative labelling of urinary, skeletal-muscle and intestinal 3-methylhistidine cannot be explained in terms of skeletal muscle accounting for a major proportion of urinary 3-methylhistidine. Measurements were also made of the steady-state synthesis rate of protein-bound 3-methylhistidine in intestinal smooth muscle *in vivo* in adult female rats. This involved measurement of the overall rate of protein synthesis and measurement of the relative rates of synthesis of 3-methylhistidine and of mixed protein. The synthesis rate of 3-methylhistidine was 29.1%/day, compared with the overall rate of 77.1%/day for mixed, non-mucosal intestinal protein. Measurement of the amount of 3-methylhistidine in skeletal muscle ($0.632 \pm 0.024\,\mu$mol/g) and in the whole body ($0.332 \pm 0.013\,\mu$mol/g) indicate that, although the muscle pool is 86% of the total, because of its slow turnover rate of 1.1–1.6%/day, it only accounts for 38–52% of the observed excretion. Measurements of the mass of the intestine (9.95 g/250 g body wt.) and protein-bound 3-methylhistidine content (0.160 μmol/g of tissue) indicate a pool size of 1.59 μmol/250 μg rat. Thus 463 nmol of the urinary excretion/day would originate from the intestine, 22% of the total. The tissue source of the remaining urinary excretion is not identified, but other non-muscle sources constituting about 10% of the whole-body pool could account for this with turnover rates of only 6%/day, a much lower value than the turnover rate in the intestine.

We have previously questioned the assumption that urinary 3-methylhistidine (N^{τ}-methylhistidine) arises predominantly from skeletal muscle, on the basis of two measurements (Millward *et al.*, 1980). Firstly we reported that the turnover of skeletal-muscle 3-methylhistidine was so slow that non-muscle sources must account for the major proportion of urinary excretion. This finding was confirmed in the preceding paper (Bates *et al.*, 1983). Secondly we reported the decay curve of radioactive labelling in urinary 3-methylhistidine after pulse labelling with [*methyl*-^{14}C]methionine, showing that there appeared to be pools of very rapidly turning-over protein-bound 3-methylhistidine in the body, although, because of the possibility of persistence of labelling in the body methionine pool with continued incorporation into 3-methylhistidine, we said that the pool sizes could not be determined accurately.

We have not identified the non-muscle sources, but the smooth muscle of the intestine is a likely candidate. We showed in one series of measurements that the turnover rate of 3-methylhistidine in this tissue was considerably faster than in skeletal muscle, so that its contribution to urinary 3-methylhistidine was about half that of skeletal muscle

(Millward *et al.*, 1980). More recently Wassner & Li (1982) have demonstrated that the perfused hemicorpus and gastrointestinal tract of the rat release 3-methylhistidine at rates indicating that 59 and 41% of the urinary excretion of 3-methylhistidine originates from skeletal muscle and intestine respectively. These measurements indicated that turnover of 3-methylhistidine in the intestine was 24%/day, a value twice as fast as our previous report (Millward *et al.*, 1980).

We now report further experiments aimed at identifying the origin of urinary 3-methylhistidine. We have extended our measurements of the decay of label in urinary 3-methylhistidine by following the labelling in muscle and intestinal 3-methylhistidine in an attempt to understand better the kinetics of 3-methylhistidine turnover. These results indicate substantial non-skeletal-muscle sources and also confirm a much faster turnover of intestinal 3-methylhistidine compared with that of skeletal muscle, which we have quantified with additional measurements of intestinal 3-methylhistidine synthesis. We have also measured the concentration of 3-methylhistidine in the whole body, skeletal muscle and intestine so that we can more confidently determine the contribution of skeletal muscle and intestine to urinary 3-methylhistidine synthesis. We have published a preliminary account of some of this information previously (Bates & Millward, 1981).

Experimental

Animals

The rats used in this study were albino CD:COBS (Charles River, Margate, Kent, U.K.), which were fed *ad libitum* with a purified diet free of 3-methylhistidine, in which the protein source was casein, comprising 20% (w/w) of the diet.

Decay of 3-methylhistidine in tissues and urine

For this, 24 female rats weighing 247 ± 6 g were divided into six groups of four. All were injected intraperitoneally with L-[*methyl*-^{14}C]methionine (50 Ci/mol; 30.2 μCi in 0.25 ml of 0.9% NaCl), and one group was put into metabolic cages for urine collection. The urine for the first 6 h was discarded and that of the next 18 h was collected into a flask containing 1 ml of 6 M-HCl, then 24 h collections were made at the times specified in the Results section. The urine volume for each collection was measured, and an equal volume of concentrated HCl was added to give a concentration of 6 M-HCl, and the urine was hydrolysed at 110°C overnight. The amount and specific radioactivity of 3-methylhistidine in the urine were measured in the hydrolysate as described below. Groups of rats were killed on days 2, 5, 15, 30, 45 and 60, the final group being those from which urine had been collected in the metabolic cages. Hind-limb muscle and intestine were dissected from each rat, the mucosa was quickly removed from the rest of the intestine by scraping with a glass slide and the tissues were frozen in liquid N_2 until analysis. The tissues were homogenized in 1.5 vol. of cold 5% (w/v) sulphosalicylic acid/10% (v/v) ethanol/0.5% thiodiglycol. The supernatant from muscle was analysed for *S*-adenosylmethionine specific radioactivity as described previously (Bates *et al.*, 1983). The protein pellets after being washed twice with sulphosalicylic acid were hydrolysed in 6 M-HCl and analysed for 3-methylhistidine specific radioactivity as described previously (Bates *et al.*, 1983).

Urinary 3-methylhistidine analyses

The pyridine elution from cation-exchange resin was done by a batch method instead of the column method described previously (Bates *et al.*, 1983). About 8 ml of AG 50 X8 cation-exchange resin (Bio-Rad Laboratories, Watford, Herts., U.K.) in a 20 ml screw-stoppered tube was washed with about 12 ml each of 0.5 M-NaOH, water, 0.5 M-HCl and water, by inverting and mixing for about 30 s each time, centrifuging in a bench centrifuge and aspirating the waste solution. The resin was then equilibrated with 2×12 ml of 0.2 M-pyridine and the sample added. The urine sample was either added directly in the 6 M-HCl or the HCl was removed by evaporation to dryness under vacuum and the sample redissolved in 0.2 M-pyridine. Generally 4–8 ml of sample was used, and the volume added was made up to 12 ml with 0.2 M-pyridine. The sample and resin were mixed for at least 5 min before the resin was centrifuged down in a bench centrifuge and the supernatant was aspirated. The resin was then washed with 8–15 vol. of 0.2 M-pyridine, until the brown contaminant was removed, the larger volumes being required when the sample was added in HCl. The 3-methylhistidine was then eluted with 1 resin volume of 2 M-pyridine, and this was evaporated to dryness and loaded on to the Locarte amino acid analyser as described previously (Bates *et al.*, 1983). The resin was washed with 5 M-pyridine and re-equilibrated with 0.2 M-pyridine for re-use. The recovery from this method was variable and only 10–20%, but, since only specific radioactivities were required, this was sufficient and samples could be processed faster than on columns. The specific radioactivities measured by this technique were found to be identical with those measured after pyridine elution from columns.

Quantification of 3-methylhistidine in the hydrolysed urine was done on a small sample run on a Chromaspek amino acid analyser (Rank–Hilger, Margate, Kent, U.K.) with detection by *o*-phthalaldehyde, where 3-methylhistidine could be successfully separated from contaminants.

Measurements of intestinal 3-methylhistidine synthesis

This was done on a group of six adult female rats, by a combination of measurements of the overall rate of synthesis with the large-dose method of Garlick *et al.* (1980), involving [^{3}H]phenylalanine, and of the relative synthesis rates of histidine and 3-methylhistidine with the single injection of [^{14}C]-histidine (Bates *et al.*, 1983). L-[U-^{14}C]Histidine (300 Ci/mol; 42 μCi in 0.2 ml per rat) was injected into a tail vein $4\frac{1}{2}$h before the animal was killed, and 10 min before killing L-[4-^{3}H]phenylalanine (50 μCi/ml; 150 mM-phenylalanine; 2.5 ml per rat) was injected into the tail vein. The animals were killed by decapitation and blood was collected into heparinized tubes. The entire gastrointestinal tract was dissected out, cooled in ice/water and the mucosal layer was removed by scraping with a glass microscope slide, leaving the remainder for analysis. From this, tissue intracellular and protein-bound phenylalanine were isolated and specific radioactivities were measured, together with those of the plasma and injection solution, as in Garlick *et al.* (1980). Protein-bound histidine and 3-methylhistidine were separated from other amino acids on a Dowex 50 X8 column (0.7 cm × 8.5 cm) with pyridine buffers, and their specific radioactivities were measured on a Locarte amino acid analyser with a split-stream facility. Total protein-synthesis rate (K_{s}) was calculated from protein-bound (S_{b}) phenylalanine specific radioactivities from the equation:

$$K_{s} = \frac{S_{B}}{\overline{S}_{i} \cdot t}$$

where $\overline{S}_{i}$ is the mean of the injection-solution and the intracellular specific radioactivities. 3-Methylhistidine synthesis was calculated on the assumption that the incorporation into histidine was proportional to the overall rate, so that the 3-methylhistidine synthesis rate was equal to the overall rate multiplied by the ratio of 3-methylhistidine labelling to that of histidine.

Determination of urinary excretion, muscle, intestinal and whole-body 3-methylhistidine

Six non-growing rats weighing approx. 260 g were maintained on the methylhistidine-free purified diet, and urine was collected in metabolism cages each day for 5 days. After this time the rats were killed with chloroform, mixed hind-limb-muscle was removed and weighed and the rest of the carcass was stored at −20 °C until analysis. The muscle sample was rapidly minced with scissors on an ice-cold glass plate, and then homogenized in cold 10% (w/v) trichloroacetic acid. After two more washings with cold 10% acid, the protein pellet was hydrolysed in 6 M-HCl at 110 °C in sealed tubes for 16 h. The 3-methylhistidine in this hydrolysate was measured by two methods. The first method was on a Rank–Hilger Chromaspek amino acid analyser. Initially, the very large histidine peak tended to swamp the small 3-methylhistidine peak. However, it was found that, if 2.5% formaldehyde was introduced into the line before reaction with *o*-phthalaldehyde, this decreased the histidine fluorescence by about 80% and actually increased the 3-methylhistidine fluorescence slightly.

The second method used high-pressure liquid chromatography of a fluorescent derivative of 3-methylhistidine, based on the method of Jones *et al.* (1982). The sample was made to form a derivative with fluorescamine, and 100 μl was run on a column (0.6 cm × 12 cm) of Partisil 10 ODS 3 reverse-phase resin (Whatman, Maidstone, Kent, U.K.). 3-Methylhistidine was eluted with a single buffer of 45% (v/v) methanol at about 7 min. The two methods gave identical results.

Each carcass was chopped as finely as possible with a guillotine, while still frozen, into 1-litre plastic centrifuge flasks. Then 700 ml of cold 10% trichloroacetic acid was added to each and the carcasses were homogenized with a large Polytron homogenizer (Northern Media Supplies Ltd., North Cave, North Humberside, U.K.). After centrifuging at 2800 ***g*** for 30 min, the supernatant was removed and the precipitate dissolved in a known volume of 1 M-NaOH at 50 °C for 2 h. A measured quantity of this solution was removed, HCl was added to give a final concentration of 6 M and the protein hydrolysed at 110 °C for 16 h. The 3-methylhistidine content of this hydrolysate was measured both with the Chromaspek amino acid analyser, with formaldehyde included in the line, and by the reverse-phase chromatography of the fluorescent derivative, as above, and identical results were obtained by both methods.

Measurements of the weight and 3-methylhistidine content of the intestine were made on a separate but similar group of rats. The entire gastrointestinal tract was removed and the contents were washed out with ice-cold water. After weighing, the protein was precipitated in cold 10% trichloroacetic acid, washed and hydrolysed in 6 M-HCl for 24 h at 110 °C in a screw-top tube. 3-Methylhistidine was determined in the hydrolysate by ion-exchange chromatogrpahy as described above.

Results and discussion

Decay curves of urinary and tissue 3-methylhistidine

The decay of radioactivity in urinary 3-methylhistidine is shown in Fig. 1. Although the rats in this experiment were older and heavier than those in our previous experiment (Millward *et al.*, 1980), the

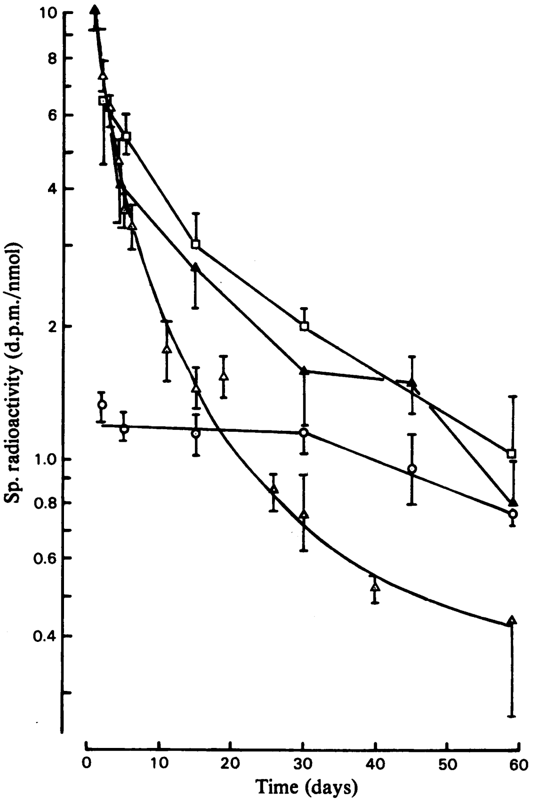

Fig. 1. *Decay of specific radioactivity in 3-methylhistidine isolated from intestinal smooth-muscle protein* (□), *skeletal-muscle protein* (○) *and urine* (△), *and skeletal-muscle S-adenosylmethionine* (▲)

Values shown are means ± 1 S.D. from groups of four adult female rats injected with [*methyl*-^{14}C]methionine on day 0.

shape of this decay curve is very similar to the one that we previously reported. Furthermore, if the curve is stripped into three exponentials by assigning a value of $0.0108\,\text{day}^{-1}$ as the rate constant for the slowest exponential, then values for the rate constants for the other two exponentials and for the tissue pool sizes from which these three components originate can be calculated from the intercepts of the three exponentials and are very similar to the values that we reported in our previous paper (Millward *et al.*, 1980). However, as we argued in that paper, the quantitative analysis of the decay curve is not likely to produce accurate results because of the persistence of radioactivity in the precursor pool. This will markedly slow down the rate of decay of the specific radioactivity. The extent to which persistence of radioactivity in the precursor pool actually occurs is demonstrated by the other results in Fig. 1, which show the fall in labelling of *S*-adenosylmethionine and 3-methylhistidine in skeletal muscle throughout the experiment. The precursor labelling was greater than that of the protein-bound 3-methylhistidine in muscle for at least 30 days after the injection, and consequently there was no fall in the labelling of the product during this time. The persistence of label in the *S*-adenosylmethionine pool no doubt reflects the recycling of the methyl groups of methionine through the C_1 pool.

A comparison of the labelling of the urinary 3-methylhistidine with that of 3-methylhistidine in skeletal muscle and intestine should give information about the extent to which these two tissues could contribute 3-methylhistidine to the urine. As shown in Fig. 1, the specific radioactivity of protein-bound 3-methylhistidine in the intestine was initially much greater than that in skeletal muscle and fell during the course of the experiment, as previously reported by Nishizawa *et al.* (1977*b*). This suggests that 3-methylhistidine in the intestine turns over more rapidly than in muscle, although without information about the time course of the labelling of *S*-adenosylmethionine in this tissue the actual turnover rate cannot be evaluated from these data.

The labelling of urinary 3-methylhistidine decayed much more rapidly than did protein-bound 3-methylhistidine in either skeletal muscle or intestine. At 15 days urinary 3-methylhistidine had only half the labelling of intestinal 3-methylhistidine, and after 30 days urinary 3-methylhistidine labelling was less than that in either skeletal muscle or intestine. It is therefore difficult to account for the urinary 3-methylhistidine-decay curve by the decay of 3-methylhistidine in either skeletal muscle or intestine. There are two possible explanations of the shape of the decay curve. The first was suggested by Ward & Buttery (1979), who offered the explanation that the shape of this curve (which they also reported) resulted from non-random degradation of muscle myofibrillar protein. Thus, if newly synthesized (highly labelled) proteins were preferentially degraded, the decay curve would be more rapid than otherwise. The effect of such a mechanism would be to limit the increase in labelling of protein at early times, when the precursor was more highly labelled than the product. In the present experiments, the *S*-adenosylmethionine labelling was greater than the labelling of protein-bound 3-methylhistidine for 50 days after the injection (Fig. 1), and during the first 15 days *S*-adenosylmethionine was 3–8 times more highly labelled than 3-methylhistidine. If the labelling of the *S*-adenosylmethionine as measured is really that of the actual precursor, then the product labelling should have increased until a precursor–product crossover occurred (Zilversmit *et al.*, 1943). Since this did not occur, it may be that Ward & Buttery (1979) are correct and that non-random degradation of muscle protein-bound 3-methylhistidine occurs. However, although this might account for the steep decay in the early part of the urinary decay curve, it cannot account for the fact that, by 30 days, the urinary 3-methylhistidine was much less highly labelled than that of muscle. This is because any first-in/first-out mechanism of degradation of muscle protein would liberate 3-methylhistidine which was labelled to an extent between the observed values for *S*-adenosylmethionine and protein-bound 3-methylhistidine which would have been substantially higher than the observed labelling in urine.

Whether intestinal 3-methylhistidine turnover is responsible for the urinary decay is not clear from these data. If the time course of *S*-adenosylmethionine labelling in the intestine is similar to that in muscle, then intestine would produce 3-methylhistidine more highly labelled than urinary 3-methylhistidine whatever the mechanism of degradation of the contractile proteins for the duration of the experiment. If, on the other hand, intestinal *S*-adenosylmethionine labelling fell to much lower values than in muscle, which might be expected on the grounds that intestinal methionine was better mixed with dietary unlabelled methionine, then a first-in/first-out non-random degradation process could account for 3-methylhistidine with the labelling observed in the urine.

The second explanation for the shape of the decay curve, which would follow if protein degradation is random, is that there is a major source of urinary 3-methylhistidine which is not muscle or intestine and in which turnover of both *S*-adenosylmethionine and 3-methylhistidine is so rapid that, by 25 days, their labelling is substantially less than in either muscle or intestine. Assuming that muscle and intestine account for at least half the excreted 3-methylhistidine, then at 60 days the rest of the urinary 3-methylhistidine must originate from a source which is virtually unlabelled or at the most has only 10% of the labelling of muscle or intestine.

Clearly, without information about the kinetics of protein degradation, it is not possible to distinguish between these different interpretations of the decay curve. However, the latter part of the curve can only be explained in terms of either intestinal 3-methylhistidine liberated by a non-random first-in/first-out mechanism accounting for a significant portion of the urinary pool, or by a pool which turns over much more rapidly than that in intestine and liberates 3-methylhistidine by a random degradation process. Neither possibility allows for skeletal muscle to be the major source of urinary 3-methylhistidine.

Intestinal 3-methylhistidine turnover

The measurement of protein synthesis in the intestinal submucosal protein in these experiments is reliable, since the technique of flooding with the large dose of labelled phenylalanine results in an expanded intracellular pool of phenylalanine, which was 92.2 ± 4% of the labelling of phenylalanine in the plasma (Table 1). Thus the real precursor labelling of the phenylalanine was likely to have been close to the measured value. Furthermore, since the tissue labelling after 10 min was 87% of the initial labelling (i.e. the injection solution), the assumption that the average labelling of the precursor over the 10 min was the mean of the initial and final values could not have involved a significant error. The measurement of the relative labelling of 3-methylhistidine and histidine in the intestinal protein is similarly likely to be a reliable indication of the synthesis rates of 3-methylhistidine as a proportion of the overall rate, since the 4½ h period over which the incorporation was measured would probably preclude any time-phase differences between peptide-bond synthesis and histidine methylation affecting the results. In any case, as we have previously argued (Bates *et al.*, 1983), other reports indicate that histidine methylation is an early post-translational event tightly coupled to translation (Watkins & Morgan, 1979).

Table 1. *Turnover of mixed protein and 3-methylhistidine in gastrointestinal tract*

Measurements were made on 250 g female rats. Measurements of the synthesis rate of mixed protein isolated from the gastrointestinal tract (without the mucosal layer) were made by assessing the incorporation of [^{3}H]phenylalanine, given intravenously as a large flooding dose over 10 min (Garlick *et al.*, 1980) as described in the text. Measurements of the synthesis rate of protein-bound 3-methylhistidine in the same animals were made by determining the incorporation over 4½ h of a trace amount of [^{14}C]-histidine into both histidine and 3-methylhistidine isolated from the same protein hydrolysate as that analysed for phenylalanine. The incorporation into histidine was assumed to be proportional to the overall rate of protein synthesis, so that the incorporation into 3-methylhistidine as a percentage of the incorporation into histidine was assumed to be equal to the synthesis rate of 3-methylhistidine as a percentage of the overall rate of protein synthesis. Values shown are means ± 1 S.D. for six rats.

Phenylalanine labelling (d.p.m./nmol)	
Injection solution	751
Plasma	725 ± 11
Intracellular	657 ± 34
Protein	3.87 ± 0.54
Synthesis rate (%/day)	76.8 ± 8.7
Histidine labelling	
3-Methylhistidine/histidine ratio	0.369 ± 0.40
3-Methylhistidine synthesis (%/day)	29.1 ± 1.38

Table 2. *3-Methylhistidine turnover and contribution of skeletal muscle and gastrointestinal tract to urinary excretion*
Values shown are for 250 g adult female rats maintained on a 3-methylhistidine-free diet for at least 3 days before measurement. Urinary 3-methylhistidine excretion is the overall mean value for all rats measured for each of 5 days. Daily coefficient of variation was 16%, and between-rats variation was 9%. 3-Methylhistidine in muscle and whole-body protein was measured by ion-exchange and reverse-phase chromatography as described in the text. Muscle pool size is calculated by assuming muscle to account for 45% of body weight. Whole-body turnover is calculated from the urinary excretion and whole-body content. Skeletal-muscle turnover includes two values: A is from Millward *et al.* (1980) measured by constant infusion of [^{14}C]methionine, and B is calculated from combined measurements of the overall rate of synthesis by [^{14}C]tyrosine infusion in the same rats as for A (Brown *et al.*, 1981) and measurements of the relative synthesis rates of histidine and 3-methylhistidine after trace labelling with [^{14}C]-histidine in adult female rats reported in Bates *et al.* (1983). Intestinal 3-methylhistidine turnover is the value derived in Table 1. Excretion is calculated as the product of pool size and turnover rate. Directly measured values are shown as means ± 1 S.D. for six rats.

Tissue	Pool size (μmol)	Turnover rate (%/day)	Urinary excretion (μmol/day)	Urinary excretion (% of total)
Whole body				
Per g	0.332 ± 0.013			
Per 250 g rat	83.0 ± 3.3	2.54	2.11 ± 0.19	100
Skeletal muscle				
Per g	0.632 ± 0.024			
Per 250 g rat	71.1	A 1.13	0.80	38
		B 1.55	1.10	52
Intestine				
Per g	0.160 ± 0.010			
Per 250 g rat	1.59 ± 0.10	29.1 ± 1.4	0.463	22

The rate of intestinal protein turnover is high, our value of 77%/day being somewhat higher than that reported by Garlick *et al.* (1982) for jejunal serosa. However, as those authors showed, the mucosal protein turnover rate is even faster, at 123%/day, so that differences in the technique of separation of mucosa and submucosal layers could result in differences in submucosal synthesis rate.

3-Methylhistidine synthesis, at 37% of the overall rate, i.e. 29%/day, is much faster than in skeletal muscle and much faster than observed previously by us (Millward *et al.*, 1980). In those experiments we measured the incorporation of label from *S*-adenosylmethionine into 3-methylhistidine during a 6 h constant infusion. The only explanation that we can offer for the difference is that the precursor was compartmented, so that the labelling of the *S*-adenosylmethionine methylating the histidine in actin was less than the measured total pool. However, we have no evidence that this was so.

Wassner & Li (1982) calculated a value for intestinal 3-methylhistidine degradation from their results for 3-methylhistidine release from, and content of, the perfused intestine. This value was very similar to our present value. However, as discussed below, the 3-methylhistidine content of intestine reported by Wassner & Li (1982) was much higher than our value. If our value for 3-methylhistidine content is combined with their value for 3-methylhistidine release, then a rate of degradation of over 60%/day would be indicated for intestinal 3-methylhistidine. It is also possible therefore that the release of 3-methylhistidine from the perfused intestine was accelerated above the rate *in vivo*.

Whole-body and tissue 3-methylhistidine content and contribution to urinary excretion

The urinary excretion rate of 3-methylhistidine in these experiments was 2.11 ± 0.19 μmol/day per 250 g rat (Table 2), which was similar to our previously reported value of 2.24 μmol/day per 250 g rat, and similar to values reported by others (Haverberg *et al.*, 1975; Wassner & Li, 1982). It should be noted that our previous report (Millward *et al.*, 1980) included a misprint in which the actual rate of urinary 3-methylhistidine excretion expressed as a function of body weight, $1.06 \pm 0.00474 \cdot$ body wt. (g) μmol$\cdot$day^{-1}, was printed as $1.06 \pm 0.00474\,\mu$mol$\cdot$day$^{-1}\cdot$g body wt.$^{-1}$. Whole-body 3-methylhistidine was 83 ± 3.3 μmol/250 g, a value 22% higher than that reported by Nishizawa *et al.* (1977*a*). These values indicate that the turnover of whole-body 3-methylhistidine is 2.54%/day (Table 2).

The concentration in skeletal muscle (0.632 ± 0.024 μmol/g; Table 2) is equivalent to 71 μmol per 250 g rat, assuming skeletal muscle to be 45% of body weight. This means that 86% of whole-body 3-methylhistidine is accounted for by muscle. In two separate reports Nishizawa *et al.* (1977*a,b*) reported that muscle accounts for 75% and 90% of whole-body 3-methylhistidine. Haverberg *et al.* (1975) reported a value of 87.6 μmol in muscle of a 278 g rat, a value 11% higher than our value. Ward & Buttery (1979) reported a value for muscle 3-methylhistidine (0.76 μmol/g) which is 20% higher than our value and which would mean that all of the 3-methylhistidine in the body could be accounted for if muscle mass was 44% of the total. The most recent report is that of Wassner & Li (1982), in which, according to their interpretation of their measurements, muscle contains 0.695 μmol/g, a value 9.9% higher than ours.

The extent to which 3-methylhistidine derived from skeletal muscle contributes to the urinary excretion can be calculated from its turnover rate. In Table 2, two values are indicated. The first (value A, 1.13%/day) was obtained in female rats (similar to those analysed in the present studies) by constant infusion of [*methyl*-^{14}C]methionine. The original value reported by us (1.08%/day) was revised to the slightly higher value when we analysed subsequent samples from those experiments. In those experiments we infused simultaneously [^{14}C]tyrosine, obtaining an overall synthesis rate of 4.18%/day, which was reported elsewhere (Brown *et al.*, 1981). This value was used together with the ratio of labelling of histidine and 3-methylhistidine (0.37), obtained after a single injection of histidine in adult female rats (Bates *et al.*, 1983) to determine the second estimate of 3-methylhistidine turnover in muscle, 1.55%/day. Since we have no reason to believe that either of these two values is in error, we feel obliged to report them both. They indicate that skeletal muscle accounts for between 38 and 52% of the urinary excretion. The validity of these values for 3-methylhistidine turnover in skeletal muscle is discussed in the preceding paper, where evidence is presented showing that actin turnover in muscle is particularly slow (Bates *et al.*, 1983). The main possible source of error in value A is that *S*-adenosylmethionine was compartmented so that we were overestimating the labelling of the precursor for methylating proteins. This possibility led us to devise the second method of histidine labelling, where precursor compartmentation is avoided. In this case we assume histidine incorporation to be equivalent to the overall rate measured with tyrosine (4.18%/day; see Brown *et al.*, 1981). This assumption is, we consider, justified, since we have been unable to observe differences in rates of muscle protein synthesis with methionine and tyrosine in the same animals (Bates *et al.*, 1983), and measurements in our Department with lysine, leucine, phenylalanine and valine have consistently given similar values for synthesis rates in the same muscle. We recognize, however, that the true rate of protein turnover in terms of overall rates of peptide-bond synthesis need not be the same as that measured by the incorporation of any single labelled amino acid. The turnover measurements are made on the gastrocnomius and quadriceps muscles, which contain mixed populations of muscle fibre types and which as far as we know have turnover characteristics representative of the rest of the skeletal-muscle mass. With the exception of the soleus and diaphragm, we have not found marked differences between turnover rates in individual rat muscles (Bates & Millward, 1983), possibly because most of them contain mixed populations of fibre types.

We have no reason to believe therefore that our value of between 38 and 52% of urinary 3-methylhistidine arising from skeletal muscle is seriously in error. We would emphasize that in our previous report (Millward *et al.*, 1980) the magnitude of the slow-turning-over pool calculated from the urinary decay curve (75%) similar to that in Fig. 1 is not likely to be in any way accurate (as we stated in that report). For this reason we have not presented the values obtainable from an analysis of the current results.

The concentration of 3-methylhistidine in intestine (160 ± 10 nmol/g) together with the intestinal mass (3.98 ± 0.21 g/100 g body wt.) are equivalent to a total pool size of 637 nmol/100 g, or about 2% of the total pool. We previously used a combination of values from the literature to arrive at a value that was 50% higher than this measured value. However, Wassner & Li (1982) reported that both the intestinal weight and 3-methylhistidine concentration were higher than our values, to the extent that the intestinal pool size that they found was over twice as large as reported here. Part of this difference may reflect the fact that their rats were a little younger than ours, since we have observed intestinal mass to be much higher in younger rats (i.e. 12% of body weight in 75 g rats). However, their value for the 3-methylhistidine concentration of intestine, 72% of that of skeletal muscle, was surprisingly high given the presence in intestine of considerable mucosal protein and connective tissue, which would not be expected to contain high concentrations of 3-methylhistidine. Our value for the concentration of 3-methylhistidine in intestine is only 25% of that in skeletal muscle (Table 2).

The product of pool size and turnover rate of intestinal 3-methylhistidine indicates, as shown in Table 2, an output of 0.463 μmol of 3-methylhistidine/day, 22% of the total. This is lower than the value (41%) reported by Wassner & Li (1982). Our value is unlikely to be a marked underestimate, since the synthesis rate measured in these fed rats is unlikely to be less than the degradation rate. On the other hand, the observed release from the perfused intestine could be an overestimate, since most perfusion systems are in negative balance, often with elevated rates of protein degradation.

It is clear therefore that intestine does contribute substantial 3-methylhistidine to the urine. Furthermore, this contribution could be much greater in young rats, since, as mentioned above, our measurements indicate that in 75 g rats intestine accounts for 12% of body weight. This difference, together with the smaller muscle mass in younger rats (Miller, 1969), means that the contribution from intestine could be much higher. This would also be the case in patients with decreased muscle mass, such as those suffering from Duchenne muscular dystrophy (Rennie *et al.*, 1982).

In the present study the estimate of the contribution from the intestine (22%) added to our highest estimate of skeletal muscle's contribution (52%) leaves 26% unaccounted for, and the source of this is currently unknown. According to Wassner & Li (1982), although there is substantial 3-methylhistidine in skin it is not a major source of urinary 3-methylhistidine, since the removal of skin from the perfused hemicorpus has little effect on 3-methylhistidine release. Our own measurements of 3-methylhistidine turnover in skin indicated that it was not much faster than in skeletal muscle. Thus skin may not be a major source. On the other hand there is considerable smooth muscle in lung and in the vascular system, and, although the total amount is not large, if the turnover is of the same order as observed here in intestinal muscle then it could account for most of the remaining excretion.

Clinical implications

The confirmation of our previous conclusion that urinary 3-methylhistidine is derived to a substantial degree from non-muscle sources means that the urinary excretion rate cannot be interpreted as a specific index of skeletal-muscle protein degradation in the rat. The extent of the involvement of non-muscle sources in normal man remains to be properly evaluated, but in patients with markedly decreased muscle mass (Duchenne muscular dystrophy) we have shown that the disparity between measured muscle protein turnover and the ratio of 3-methylhistidine and creatinine excretion rates is so marked that the muscle degradation rate, which is indicated to be increased according to the 3-methylhistidine-excretion data, is in fact decreased according to direct measurements (Rennie *et al.*, 1982). Clearly, in this particular case, the markedly decreased muscle mass resulting in a markedly increased proportion of urinary 3-methylhistidine deriving from non-muscle sources results in an elevated 3-methylhistidine/creatinine ratio.

Afting *et al.* (1981) have reported details of the urinary 3-methylhistidine excretion in a patient in which skeletal muscle was reportedly completely absent after a motor-neuron degeneration. The excretion rate was 0.069 mmol/24 h, compared with values of 0.24 ± 0.095 in controls. Although the authors stated that this showed that non-skeletal-muscle sources accounted for 28% of the excretion, it should be noted that, if account is taken of the variability in normal subjects (e.g. 1 S.D.), then the output in the patient was between 20 and 50% that of controls, implying that skeletal muscle accounts for 50–80% of urinary 3-methylhistidine excretion.

If non-muscle sources of urinary 3-methylhistidine are a significant proportion, then clearly measurement of urinary excretion alone does not allow the determination of which tissue source is responsible for any changes which are observed. In this case, arterio-venous measurements of 3-methylhistidine release across limbs are probably the only way of unequivocally determining changes in skeletal muscle protein degradation. Lundholm *et al.* (1982) and K. Lundholm & M. J. Rennie (personal communication) have performed such measurements in a variety of patients and have observed several instances of decreased output of 3-methylhistidine from the leg in catabolic situations when urinary 3-methylhistidine excretion is increased. Although such studies do not indicate the source of the non-muscle 3-methylhistidine, they do indicate that urinary excretion of 3-methylhistidine cannot be taken as an unequivocal indication of protein degradation in skeletal muscle.

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